Kidney International, Vol. 42 (1992), pp. 41–45

Mechanism of glycine protection in hypoxic injury: Analogies with glycine receptor

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Mechanism of glycine protection in hypoxic injury: Analogies with glycine receptor. Addition of glycine to the recirculating perfusate of isolated perfused rat kidneys protects against hypoxic injury to the medullary thick ascending limb and slows functional deterioration in the course of perfusion. This effect is dependent on dose; the earliest significant protection is seen at 0.25 mM, and the protective effects increase as glycine concentration is increased to 2 mM, the highest level tested. Two specific agonists of the strychnine-insensitive (NMDA) glycine receptor in neural membranes, l-aminocyclopropane carboxylic acid (ACC) and d-serine, also exerted a cytoprotective effect at a concentration of 2 mM. On the other hand, l-serine and taurine, ineffective agonists of the NMDA-glycine receptor but effective agonists of the strychnine-sensitive glycine receptor, had no protective effect in this system. Two antagonists to glycine at its binding site on the N-methyl-D-aspartate (NMDA) receptor, 7-chlorokynurenic acid (2 mM) and indole-2-carboxylic acid (12.5 mM), did not reverse the cytoprotective action of 0.25 mM glycine. The data are consistent with a ligand-acceptor type of interaction to account for cytoprotection. The configuration of the glycine acceptor may resemble, but is not identical with, that of certain glycine receptors in the nervous system.

Glycine appears to blunt or prevent cellular injury in a number of experimental models. In dispersed proximal tubules of rabbit kidneys, glycine reduces damage caused by hypoxia [1–4], metabolic inhibitors [5] or ouabain [6]. Similar protection against injury caused by cold ischemia or metabolic inhibitors has been observed in cultured hepatocytes [7, 8]. In isolated perfused rat kidneys, addition of 2 to 5 mM of glycine to the recirculating perfusate greatly diminishes functional deterioration, biochemical signs of proximal tubular injury [9], and morphological evidence of hypoxic damage to the medullary thick ascending limb [10]. The cytoprotective property of glycine does not seem to be related to its metabolism [9, 11, 12], to the maintenance of cellular levels of ATP or calcium [13, 14], or to the ability of glycine to conjugate with fatty acids via glycine-N-acyl transferase [11].

Similar though less marked protection is provided in dispersed proximal tubules of the kidney by other small amino acids, including d-alanine, l-alanine, beta-alanine, and the synthetic glycine analogue, l-amino-cyclopropane carboxylic acid (ACC) [3], suggesting a structural requirement for cytoprotection. Glycine receptors are present in the central nervous system, where two types have been identified. One, a strychnine-sensitive glycine receptor, controls the gating of chloride channels and inhibits neural excitation. Partial agonists for this receptor include l-alanine, d-alanine, beta-alanine, taurine and l-serine [15]. A second receptor site with high binding affinity for glycine modulates activity of the N-methyl, d-aspartate (NMDA) excitatory receptor [16]. Unlike the first type, it preferentially binds d-serine and ACC, and is insensitive to strychnine.

In the present experiments in isolated perfused rat kidneys, we investigated the relationship of the dose of glycine to the cytoprotection seen in this system and examined the protective effect of various agonists and antagonists of neural glycine receptors.

Methods

Male Sprague-Dawley rats weighing 300 to 320 g and allowed free access to water were used for all experiments. Isolated perfusion of the right kidney was performed according to the method of Ross, Epstein and Leaf [17]. The perfusion medium consisted of a Krebs-Ringer-Henseleit solution containing (in mM): Na 143; K 4.5; bicarbonate 24; Ca 2.5; Mg 1.2; phosphate 1.2; pH 7.4 when gassed with 5% CO_2, 95% O_2; and bovine serum albumin at a concentration of 6.7 g/dl. All perfusions were carried out for 90 minutes. Urine collections and perfusate samples were obtained for clearance measurements every 10 minutes after 30 minutes of stabilization.

Kidneys were perfused at a constant pressure of 100 mm Hg at the catheter tip. The rate of perfusate flow was monitored by a Brooks flowmeter in line. Glomerular filtration rate was estimated from the clearance of ^3H inulin (New England Nuclear, Boston, Massachusetts, USA). Fractional sodium reabsorption was calculated from the concentration of Na^+ in the perfusate and urine, and the inulin clearance. Lactic dehydrogenase (LDH) and glucose concentrations were determined in samples of urine and perfusate by automated enzymatic analysis. LDH release was expressed as the amount of enzyme appearing in urine and perfusate during the last hour of perfusion.

Morphological techniques

The morphology of the kidneys in each group was examined. A three-way stopcock was incorporated into the circuit 5 cm

Received for publication December 11, 1991
and in revised form January 30, 1992
Accepted for publication January 30, 1992.

Table 1. Functional data in kidneys perfused with various concentrations of glycine

		Control	Glycine				
Glycine concentration							
mM		0	0.1	0.25	0.5	1	2
n		(16)	(5)	(5)	(4)	(5)	(7)
Perfusate flow	30′	22.9 ± 0.5	25.7 ± 5	23.0 ± 0.8	23.7 ± 0.7	22.4 ± 6	24.7 ± 1.4
ml/min/g	60′	25.4 ± 0.7	29.4 ± 6	24.8 ± 1.4	25.1 ± 0.8	24.6 ± 3	29.0 ± 1.3
wet wt of kidney	90′	27.9 ± 1	30.7 ± 5	25.9 ± 1.0	26.4 ± 1.1	26.0 ± 6.0	31.3 ± 1.1
Urine volume	30′	66 ± 7	54 ± 2	50 ± 3	47 ± 3	72 ± 8	49 ± 7
ml/min/g	60′	116 ± 15	96 ± 2	78 ± 2	85 ± 6	114 ± 12	105 ± 9
wet wt of kidney	90′	112 ± 13	92 ± 2	86 ± 5	93 ± 10	118 ± 9	106 ± 8
Glomerular filtration rate	30′	0.49 ± 0.03	0.63 ± 0.04	0.59 ± 0.03	0.57 ± 0.05	0.49 ± 0.03	0.57 ± 0.04
ml/min/g	60′	0.28 ± 0.02	0.33 ± 0.02	0.33 ± 0.03	0.37 ± 0.03	0.39 ± 0.04	0.47 ± 0.04
wet wt of kidney	90′	0.23 ± 0.02	0.25 ± 0.01	0.26 ± 0.02	0.32 ± 0.07	0.35 ± 0.04	0.41 ± 0.04
Tubular reabsorption of sodium %	30′	94.46 ± 0.55	96.39 ± 0.39	96.23 ± 0.60	96.59 ± 0.48	93.96 ± 1.52	95.72 ± 0.77
	60′	91.03 ± 1.23	95.40 ± 0.55	96.27 ± 0.72	96.05 ± 1.24	95.3 ± 1.21	94.19 ± 0.97
	90′	90.28 ± 1.44	94.32 ± 0.99	95.31 ± 0.88	95.18 ± 1.62	95.3 ± 1.22	94.23 ± 0.88
Tubular reabsorption of glucose %	30′	94.09 ± 1.13	98.58 ± 0.31	98.98 ± 0.21			99.03 ± 0.20
	60′	77.16 ± 1.86	87.15 ± 2.49	93.26 ± 2.01			98.76 ± 0.32
	90′	68.83 ± 1.57	77.31 ± 2.21	83.89 ± 2.35			97.7 ± 0.42
LDH release during last 60 min of perfusion *units*		6.9 ± 0.4	4.2 ± 0.8	3.6 ± 0.7	1.8 ± 0.5	1.6 ± 0.3	2.1 ± 0.4

Glomerular filtration and tubular reabsorption of glucose fell significantly ($P < 0.01$) over time, while perfusate flow and urine volume increased. The rate of change over time is significantly different between groups with and without glycine for glomerular filtration ($P < 0.0001$), tubular reabsorption of sodium ($P < 0.0025$) and glucose ($P < 0.0001$, 2 way ANOVA).

from the arterial cannula to allow perfusion with the fixation solution at the same pressure applied during the functional studies for an additional five to eight minutes. The fixative solution contained 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The sections were postfixed in buffered 2% OsO_4, dehydrated and embedded in araldite-epon 812 mixture. Large 3 × 3 1 micron sections of the outer medulla were cut, stained with methylene blue and examined by light microscopy.

The histological evaluation was completed by Dr. S. Rosen in a "blinded" fashion; that is, without knowledge of the experimental condition. Three zones of the inner stripe were analyzed: Upper third (A level): All mTALs intersecting a line immediately adjacent (within 0.2 mm of) the outer stripe; middle third (B level): All mTALs intersecting a line drawn midway between the borders of the inner stripe; and a lower third (C level): All mTALs intersecting a line immediately adjacent to (within 0.2 mm of) the inner medulla. These points were chosen for analysis because they provided areas in which topographical landmarks were easily ascertained. A percentage score was used to indicate the fraction of tubules involved with moderate changes (blatant mitochondrial swelling with extensive nuclear pyknosis), or severe changes (blatant mitochondrial swelling, extensive nuclear pyknosis and cell fragmentation). Between 80 and 270 tubules (mean 130) were evaluated per kidney.

Experimental design

Dose-response relation of glycine cytoprotection. The protective action of glycine in the isolated perfused rat kidney was evaluated at concentrations of 0, 0.1, 0.25, 0.5, 1 and 2 mM glycine added to the recirculating perfusate (4 to 16 experiments per group).

Glycine receptor agonists and antagonists. The effects of glycine receptor agonists were compared to those of glycine. The agents used were glycine, l-aminocyclopropane carboxylic acid (ACC), d-serine, l-serine and taurine (5 to 7 experiments per group). Each was added to the perfusate at a concentration of 2 mM. Two antagonists to glycine at its binding site on the NMDA receptor were tested: 7-chlorokynurenic acid, 2 mM ($N = 8$), and indole-2-carboxylic acid, 12.5 mM ($N = 6$). In order to achieve the highest possible concentration of antagonists relative to that of glycine, these were tested in the presence of 0.25 mM glycine.

Statistics

Results are presented as mean ± SEM. One and two-way analysis of variance with the Neuman-Keuls test were used for comparisons between the different groups and between repeated measurements within groups. Simple correlations were performed as detailed below.

Results

Dose-dependent effects of glycine (Table 1, Fig. 1)

The structural and functional protection afforded by glycine was proportional to dose as glycine concentration was varied from 0.1 mM to 2 mM. Cytoprotection in the mTAL was most marked at the deepest (C) level of the inner stripe. Significant reduction in severe morphological injury was apparent at 0.25 mM and was pronounced at 2 mM. The inverse correlation between glycine concentration and severe injury to zone C was highly significant ($r = -0.74$; $P < 0.0001$).

Glycine concentration was directly correlated with glomerular filtration rate at 60 and 90 minutes of perfusion (Table 1, Fig. 2, $r = 0.65$, $P < 0.0001$). The release of lactic dehydrogenase (Table 1) was correlated with severe mTAL injury in zone C ($r = 0.74$, $P < 0.0001$) and inversely correlated with glycine concentration ($r = -0.78$, $P < 0.0001$). As reported earlier [10], the rate at which tubular reabsorption of sodium declined in the later stages of perfusion was slowed by glycine as compared

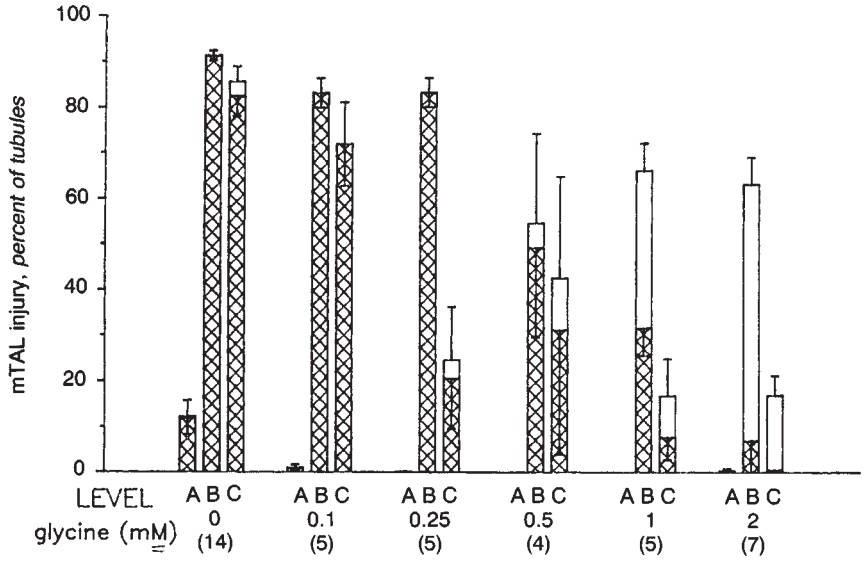

Fig. 1. *Structural damage in medullary thick ascending limbs (mTAL) of the inner stripe of the outer medulla in kidneys perfused for 90 minutes with concentrations of glycine varying from 0 (control) to 2 mM.* Zone A indicates the outer third, B the mid-level, and C the deepest portion of the inner stripe. Symbols are: (▩) severe damage; (□) moderate damage.

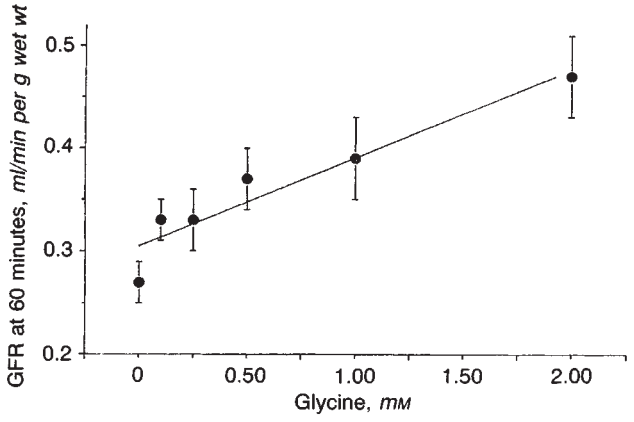

Fig. 2. *Inulin clearance (GFR) at 60 minutes of perfusion is proportional to the concentration of glycine in the recirculating perfusate* (r = *0.65;* P < *0.0001).*

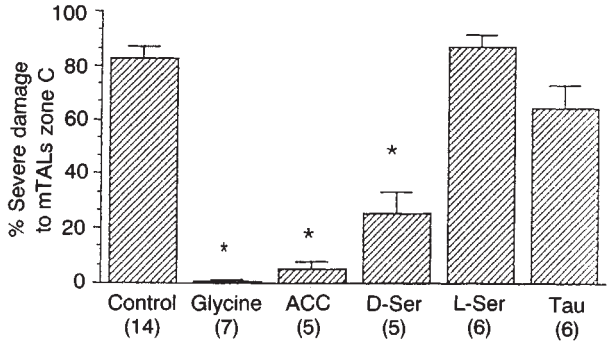

Fig. 3. *Severe structural damage to deep medullary thick limbs (mTALs) in kidneys perfused for 90 minutes with no amino acids (control), and with 2 mM glycine, l-amino-cyclopropane carboxylic acid (ACC), d-serine, l-serine and taurine.* * $P < 0.01$ versus control, ANOVA

with control perfusions, but urine volume and perfusion flow rate were not affected (Table 1). Tubular reabsorption of glucose, a function of the proximal tubule, was also improved by glycine and correlated with glycine concentration ($r = 0.4$; $P < 0.005$ at 90 min).

Glycine receptor agonists and antagonists

When equimolar concentrations of ACC or d-serine, which are glycine agonists at the NMDA receptor, were substituted for 2 mM glycine, mTAL necrosis was also substantially reduced, though not so much as with glycine (Fig. 3). As with glycine, the most pronounced effect was noted at the deepest (C) level of the inner stripe ($P < 0.01$ vs. controls). Tubular reabsorption of glucose was significantly preserved and LDH release was substantially lowered, but an ameliorative effect upon the deterioration of glomerular filtration rate and fractional sodium reabsorption was not statistically demonstrable by analysis of variance (Table 2). In contrast, l-serine and taurine, which are agonists of the strychnine-sensitive glycine receptor in the brain, but do not bind to the NMDA associated glycine receptor, did not prevent tubular damage or ameliorate renal function (Fig. 3, Table 2).

The action of glycine (0.25 mM) to prevent structural damage to the mTAL was not inhibited by addition of the glycine receptor antagonists indole-2-carboxylic acid, 12.5 mM ($N = 6$) or 7-Cl-kynurenic acid, 2 mM ($N = 8$) (Fig. 4). These agents also did not reverse the functional protection afforded by glycine on GFR, tubular reabsorption of glucose, and LDH release. When given alone in 3 experiments, indole-2-carboxylic acid (12.5 mM) reduced morphological damage in the outer stripe to approximately the same degree as 1 mM glycine. In two experiments, strychnine, 10^{-7} M, did not reverse the protective effect of glycine at a concentration of 2 mM. At higher concentrations, strychnine produced intense vasoconstriction of the perfused kidney, reducing the flow of perfusate.

Discussion

In isolated rat kidneys perfused with oxygenated, cell-free medium, cellular injury develops as the result of medullary

Table 2. Effect of glycine analogues on kidney function and LDH release in perfused kidneys

	Control (16)	Glycine 2 mM (7)	Glycine 0.25 mM (5)	ACC 2 mM (5)	d-Serine 2 mM (5)	l-Serine 2 mM (6)	Taurine 2 mM (6)
Flow *ml/min/g*	25.4 ± 0.7	29.0 ± 1.3	24.8 ± 1.4	30.5 ± 1.0	25.1 ± 0.6	30.6 ± 1.4	23.2 ± 1.1
GFR *ml/min/g*	0.27 ± 0.02	0.47 ± 0.04[a]	0.33 ± 0.03	0.41 ± 0.04	0.34 ± 0.03	0.33 ± 0.02	0.28 ± 0.03
Fractional reabsorption of sodium %	91.0 ± 1.2	94.2 ± 0.9	96.3 ± 0.72	94.6 ± 0.6	94.3 ± 0.7	83.8 ± 1.2	93.3 ± 1.4
Fractional reabsorption of glucose %	77.2 ± 1.9	98.8 ± 0.3[a]	87.2 ± 2.5[a]	93.4 ± 2.2[a]	88.3 ± 1.2[a]	75.8 ± 5.9	83.4 ± 2.6
LDH release *units*	6.9 ± 0.4	2.1 ± 0.4[a]	3.6 ± 0.7[a]	4.0 ± 0.7[b]	3.5 ± 0.6[a]	5.2 ± 0.9	5.2 ± 0.4

Values shown were obtained at 60 minutes of perfusion, with the exception of LDH release, which includes total LDH released into urine and perfusate during the last hour of perfusion; that is from 30 to 90 minutes.

[a] $P < 0.01$ versus control

[b] $P < 0.05$ versus control

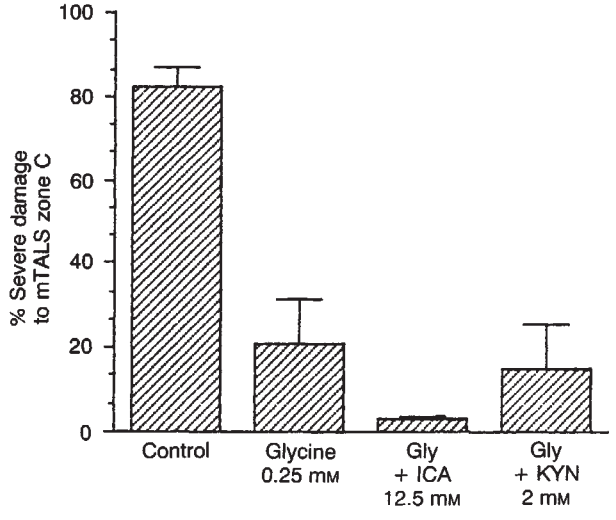

Fig. 4. *Severe structural damage to deep medullary thick limbs (mTALs) in control kidneys* (N = *16) and those perfused with 0.25 mM glycine* (N = *5), 0.25 M glycine plus 12.5 mM indole-2-carboxylic acid* (N = *6), and 0.25 M glycine plus 2 mM 7-chlorokynurenic acid* (N = *7).* The percent severe damage in control kidneys is significantly greater from that in all 3 groups of kidneys perfused with glycine ($P < 0.01$), but the latter 3 do not differ significantly from each other.

hypoxia. Morphological signs of damage are most apparent in the thick ascending limb of Henle's loop and are correlated with a decline in glomerular filtration rate and a decrease in fractional reabsorption of sodium [18]. The addition of glycine to the recirculating medium prevents morphological evidence of mTAL damage and improves kidney function [10]. While structural injury is most obvious in the medullary thick ascending limbs of isolated perfused rat kidneys, the present experiments provide evidence of functional damage to proximal tubular cells as well as in the form of a reduction in the fractional reabsorption of glucose that is preventable with glycine. Fractional glucose reabsorption was progressively depressed in control kidneys perfused without glycine, but maintained at a high level by the addition of glycine to the recirculating perfusate. These changes most likely reflect damage to the straight portion of the proximal tubule (S3), contained in the outer medulla and the medullary rays, which are known to be susceptible to hypoxic injury. Glycine also diminished the release of lactic dehydrogenase into urine and medium, an index of tubular cell injury.

In the present report we present evidence that the protective effect of glycine upon the isolated perfused kidney is dose-dependent, with significant morphological and functional preservation at a glycine concentration of 0.25×10^{-3} M. It should be noted that this concentration of glycine is considerably higher than the binding affinity of glycine receptors described in the brain, which are usually in the nanomolar range [15, 17–19].

Cytoprotection was also obtained in this model with certain glycine analogues and amino acids that bind to glycine receptors present in the central nervous system. Two types of receptors have been described. An inhibitory glycine receptor that opens a chloride channel is blocked by strychnine; its agonists include l-serine, taurine, d-alanine and l-alanine. A second glycine receptor is associated with the N-methyl-d-aspartate (NMDA) receptor, modifying it in the direction of excitation. This receptor is insensitive to strychnine but inhibited by a number of compounds, including 7-Cl-kynurenic acid and indole-2-carboxylic acid. Agonists to this receptor include d-serine and l-aminocyclopropane carboxylic acid (ACC).

In the present experiments in the isolated perfused kidney, functional and morphological integrity was maintained by ACC and d-serine, specific agonists of the strychnine-insensitive glycine receptor in neural cells. On the other hand, l-serine and taurine, which are agonists of the strychnine-sensitive glycine receptor, but do not bind to the strychnine-insensitive glycine receptor, lacked this protective effect.

A cytoprotective action of ACC was also noted by Weinberg et al in dispersed proximal tubules of rabbit kidney exposed to anoxia [3]. In that system, l-alanine, d-alanine and beta-alanine also provided some protection, while neither d-nor l-serine were effective in blunting injury. These authors suggested that "the effects of glycine analogues appear to be determined by strict requirements of molecular mimicry." Baines, Shaikh and Ho found that at a concentration of 5 mM, glycine, l-alanine, d-alanine and beta-alanine all prevented functional deterioration and release of enzymes in the isolated perfused rat kidney and that the effect of glycine was not vitiated by blocking its intermediary metabolism with cysteamine. They suggested an ability of these small molecules "to stabilize tertiary protein structure and membrane structure [9]."

It is natural to speculate that the molecular configuration responsible for the cytoprotective action of glycine and its analogues in epithelial cells might be related to that of specific glycine receptors already studied intensively in neural tissue. Evidence for this, however, is mixed. The binding constant for

glycine and related compounds in brain membranes is in the nanomolar range, while the lowest concentration of glycine found to protect kidney cells from anoxic injury is 2.5×10^{-4} M. The marked protection of mTALs in isolated perfused kidneys afforded by ACC and d-serine, both agonists of the strychnine-insensitive NMDA-associated glycine receptor, suggests a specific congruence with this receptor that is supported by the ineffectiveness of the optical isomer of d-serine (l-serine), an agonist of the strychnine-sensitive receptor. However, other agonists of the strychnine-sensitive receptor but not of the NMDA receptor, that is, l-alanine and beta-alanine, do provide cytoprotection to renal cells [3, 9, 10, 13], though taurine, which binds to the strychnine-sensitive receptor, does not, either in dispersed proximal tubules [3], or, as in the present experiments, in medullary thick limbs of the perfused rat kidney.

Two selective inhibitors of strychnine-insensitive glycine receptors were tested: 2.5 mM 7-chloro-kynurenic acid (tested at 10× the ambient glycine concentration) and 12.5 mM indole-2-carboxylic acid (tested at 50× the ambient glycine concentration). Neither was effective in reversing the cytoprotection provided by 0.25 mM glycine. In these experiments the ratio of the concentration of putative antagonist to that of glycine approximated that reported to be effective in preventing the binding of glycine to brain receptors [19, 20]. We were unable to test higher concentrations of antagonist compounds owing to their limited solubility in the perfusing medium.

In summary, the protection against hypoxic cell injury in isolated perfused kidneys that is provided by glycine is mimicked by l-amino-cyclopropane carboxylic acid and d-serine, both glycine analogues that are active on the strychnine-insensitive glycine receptor in brain membranes. By contrast, l-serine and taurine, which are inactive on this receptor, though active agonists on the strychnine-sensitive receptor, did not provide protection. Two glycine receptor antagonists in brain, however, did not reverse the glycine effect. On balance, the results of these and other studies favor a ligand-acceptor type of interaction to account for cytoprotection, rather than one involving the metabolism of glycine. The molecular configuration of the acceptor involved in glycine's cytoprotective action may bear some resemblance to certain glycine receptors in the nervous system.

Acknowledgments

This work was supported by grants from the National Institutes of Health (DK-18078, DK-39249) and the Baxter Healthcare Corporation. It was presented in part as an abstract at the national meeting of the American Society of Nephrology (*J Am Soc Nephrol* 1:595, 1990). The authors thank Lena Ellezian for assistance.

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